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Analysis of protein interactions using fluorescence technologies

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Biophotonics techniques, especially those involving fluorescence, are widely used in proteomics to characterize the *in vitro* interactions between proteins in high-throughput mode. On the other hand, fluorescence-based imaging studies often show that protein activity is regulated through large protein complexes that transiently form at specific sites in the cell. One could therefore argue that a systematic functional analysis of the human proteome requires technologies that are capable of time and spatially resolved, multiplexed analysis of protein interactions within cells.

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Abbreviations

CFP	cyan fluorescent protein
FCS	fluorescence correlation spectroscopy
FLIM	fluorescence lifetime image microscopy
FP	fluorescence polarization
FRET	Förster resonance energy transfer
GFP	green fluorescent protein
HTS	high-throughput screening
RFP	red fluorescent protein
YFP	yellow fluorescent protein

Introduction

A long-term research objective in the field of functional proteomics is to demonstrate how the spatial and temporal regulation of a specific protein complex is coupled to a specific activity and a particular cellular response (e.g. motility) [1••]. Several approaches have been proposed to achieve this goal, including high-throughput surface-based identification of interacting protein pairs within a proteome [2••], and live cell imaging of protein interactions [3]. High-throughput analysis of protein interactions is usually limited to complexes with dissociation constants of 10^{-5}–10^{-12} M, while binding measurements made in living cells will involve fewer than 10^4 protein molecules. Consequently the techniques used to quantify protein interactions must be sensitive over this concentration range. Furthermore, these techniques should be capable of identifying interactions of specific proteins against a background of more than 30 000 other proteins within a living cell. Fluorescence spectroscopy and fluorescence imaging can meet these criteria [4••]. Indeed, total internal reflection fluorescence microscopy and fluorescence correlation spectroscopy (FCS) using one- or two-photon excitation are commonly used to quantify the activity, interactions and dynamics of single protein molecules within living cells [5].

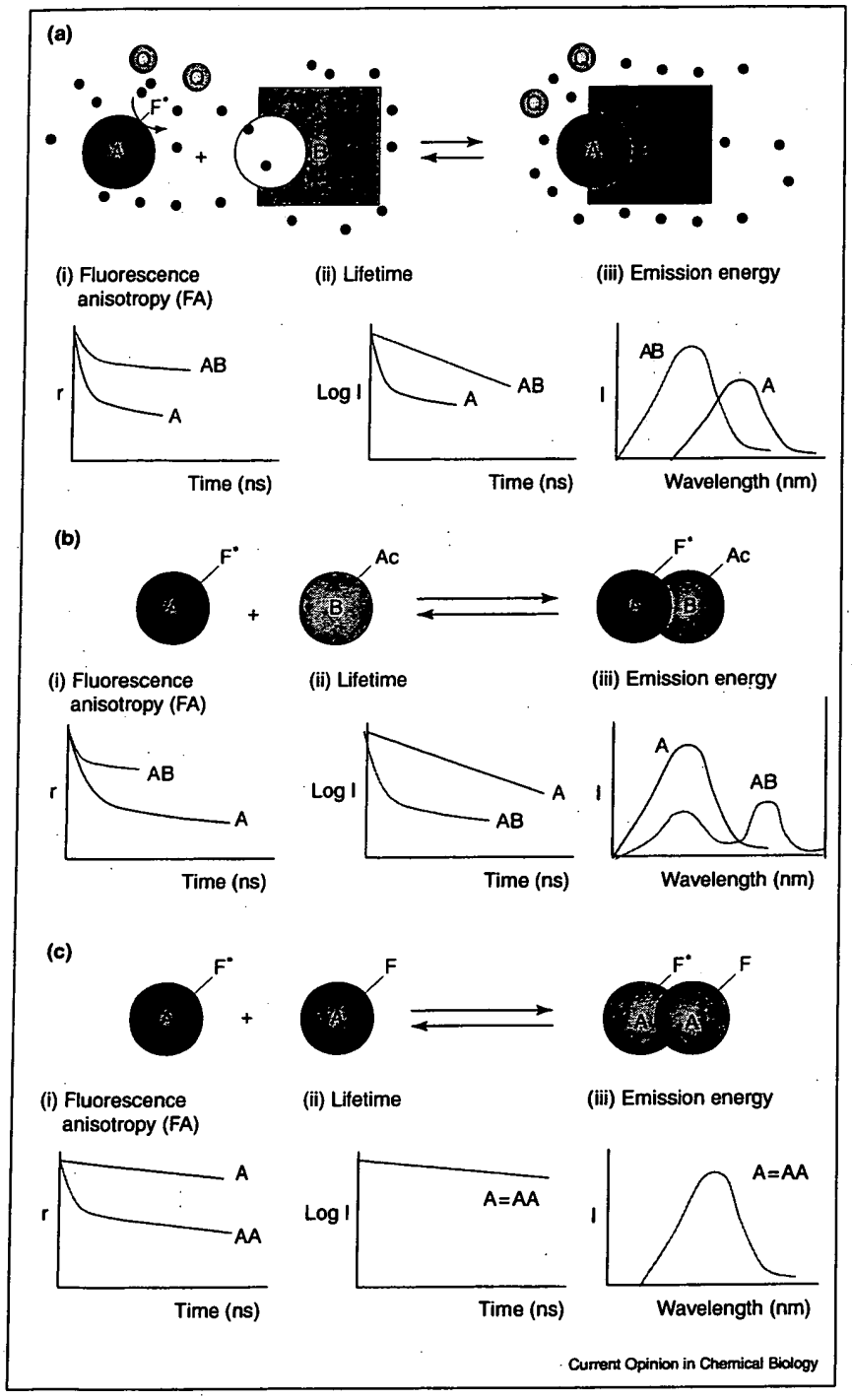
This review covers some basic principles underlying fluorescence analysis of protein interactions and highlights approaches that are used to quantify functional protein interactions *in vitro* and *in vivo* under conditions that approximate equilibrium binding. We do not cover fluorescence techniques for protein profiling, which usually involve quantifying the amount of a protein captured on a surface under conditions that approximate stoichiometric binding [2••]. Fluorescence-based approaches to study protein interactions in solution have recently been reviewed by Jameson *et al.* [4••], and reviews on fluorescence microscope techniques including FCS [6,7], Förster resonance energy transfer (FRET) imaging [3,8] and fluorescence lifetime image microscopy (FLIM; [9]) have been extensively reviewed and most recently appeared in a two-volume issue of *Methods in Enzymology* devoted to biophotonics research [10].

The molecular environment around a probe influences the fluorescence emission

Many protein interaction assays are designed around protein conjugates whose intensity changes between the free and bound states. However, relative changes in the intensity of a fluorophore cannot be accurately compared between different laboratories. This limitation can be overcome by measuring an *absolute* photo-physical parameter of the fluorescence emission. Four absolute photophysical parameters of the fluorescence emission have been used to quantify protein interactions *in vitro* and *in vivo*: polarization, lifetime, average energy and quantum yield.

These photophysical parameters can be measured using steady-state or time-resolved techniques. These techniques have also been adapted for both high-throughput and high content measurement modes [11–14]. Conditions that can lead to a change in the lifetime, average energy and polarization (or anisotropy) of a fluorescent protein conjugate are schematized in Figure 1. In reviewing fluorescence methods to quantify protein interactions we noticed that some of the most recent significant developments have been made for measurements of

Figure 1



The interaction between a fluorescently labeled protein (A–F) and a non-labeled protein (B) can be characterized through various experimental approaches. **(a)** Approaches to quantify heteromeric protein interactions using a single protein conjugate. (i) Time-resolved (or steady-state) fluorescence anisotropy (r) of the conjugate, which increases in the complex (AB) due to the increased hydrodynamic volume. (ii) Fluorescence lifetime of the conjugate, which usually increases in the complex (AB) due to a decrease in the rate of quenching effects caused by solvent (pink circles) and heavy atom quenchers (e.g. iodide, green circles) and the changes in the molecular environment around the probe. The plot depicts the decrease in the logarithm of the fluorescence intensity versus time after a picosecond pulse of excitation light in the free and bound forms of the fluorescent conjugate. (iii) Steady-state fluorescence emission spectrum of the conjugate, which changes in the complex (AB) due to the different molecular environment around the probe and the formation of new dipolar interactions between the protein and the probe. **(b)** Approaches to quantify

fluorescence lifetime and fluorescence anisotropy (FA) within the microscope rather than solution-based fluorometers and high-throughput fluorescence plate readers. The focus of our review reflects this trend.

Design of fluorescent conjugates for probing protein interactions

When designing a fluorescent conjugation for a protein interaction assay, it is usually a good idea to select a probe that can respond to a perturbation in its molecular environment by exhibiting changes in two or more of the fluorescence parameters discussed above [4••]. Ideally the probe should be covalently attached to the protein of interest through a unique labeling site (e.g. the thiol group of a single cysteine residue). We illustrate the relationship between the molecular environment of the probe at its attachment site and its fluorescence emission properties by considering IAEDANS, a small, charged and thiol-reactive fluorophore having a relatively long fluorescence lifetime. The charged sulfonate usually restricts the location of IAEDANS to the protein surface, where it interacts with solvent molecules and ions that tend to shift the wavelength of the emission to the red (lower energy). Furthermore, IAEDANS may experience considerable local motion around its attachment site during the excited state lifetime (~12 ns) that can lead to a relatively low steady state value of FA. The molecular environment around the IAEDANS can change appreciably on formation of a protein complex — for example a reduction of solvent-mediated dynamic quenching will increase the fluorescence lifetime and quantum yield (Figure 1a, ii). In addition, the probe may experience a lower dielectric environment within the larger protein complex and engage in new interactions with dipoles from the protein that displace water molecules. These combined effects will tend to shift the fluorescence emission to lower wavelength (higher energy) compared with the uncomplexed IAEDANS-conjugate (Figure 1a, iii). Finally for this particular probe, the larger hydrodynamic volume of the protein conjugate in the complex will lead to an increase in the steady state FA value (Figure 1a, i).

Genetically encoded fluorescent fusion proteins based on green fluorescent protein (GFP) and its color variants are often employed to map protein interactions within living cells. However, unlike most organic fluorophores, the GFP probe is highly immobilized within the protein matrix and is largely shielded from solvent effects. The lifetime, FA and energy of the emission of a GFP-fusion protein do not, therefore, change to any significant degree within a larger protein complex [12,15•]. Consequently approaches using fluorescent proteins to map protein interactions involve FRET measurements between two interacting proteins fused to cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), and GFP and red fluorescent protein (RFP), respectively, or to a GFP-fusion protein and an extrinsically labeled fluorophore introduced by FLASH labeling technology [16] or microinjection [17].

Fluorescence lifetime measurements and imaging

Time-resolved analysis of the emission decay from the excited state of a fluorescent probe provides an opportunity for multiplexed analysis of protein conjugates. Each component in a sample can be resolved on the basis of its decay rate. On the other hand, relatively few probes are known whose fluorescence lifetime is sensitive to changes in molecular environment [4••,18,19], and more often the decay is multi-exponential and must be averaged for the final read-out. However, the lifetime of a protein-attached probe will change appreciably and in a well-behaved fashion when it engages in FRET with an acceptor probe within the protein complex [8]. In many situations, the efficiency of FRET is more accurately measured using lifetime measurements, although in practice, and especially in the case of high-throughput screening (HTS) assays, FRET is determined through fluorescence intensity measurements of the donor or acceptor probe under steady-state conditions of illumination [20••,21]. However, we note the increasing trend in using certain lanthanide ion chelates that have μs–ms emission lifetimes as the donor probes, and red-shifted organic probes as the acceptors in the FRET measurement based on the delayed luminescence lifetime of the donor or acceptor [22].

The need for cell biologists to measure prompt (nanosecond) FRET between two probes on interacting proteins within living cells has led to the rapid development of microscopes capable of recording fluorescence lifetimes on a pixel-by-pixel basis [17,23]. FLIM [9,24], which obtains images in terms of decay rate, or lifetime, is primarily used for FRET-based imaging of protein complex within cells (see following section).

(Figure 1 Legend Continued) heteromeric protein interactions using hetero-FRET. (i) Time-resolved (or steady-state) anisotropy of the donor probe (A), which increases in the complex (AB) because of the shorter fluorescence lifetime due to energy transfer to the acceptor probe (Ac) and the larger hydrodynamic volume. (ii) Fluorescence lifetime of the donor probe, which decreases due to energy transfer to the acceptor probe during its excited state lifetime. (iii) Steady-state fluorescence emission spectrum, which decreases in intensity over the donor wavelength region and may increase over the acceptor wavelength region if the acceptor is a fluorescent probe. (c) An approach to quantify homomeric protein interactions using homo-FRET. (i) Time-resolved (or steady-state) anisotropy of the donor probe (A), which decreases in the complex (AA) because of energy transfer between two or more probes. The transfer occurs between probes that have different orientations in the complex – as a result, the emission from the 'acceptor' probe will have a lower anisotropy compared with the same probe in the uncomplexed protein. (ii, iii) The fluorescence lifetime and steady-state fluorescence emission spectrum of the probe do not change in homo-FRET.

Hetero-FRET

The excited state electron of a fluorescent probe can return to the ground state by a radiative process (fluorescence emission) and other non-radiative processes [8]. One of the most elegant methods to quantify protein interactions is measuring the rate of non-radiative transfer from the excited state of a fluorophore attached to one protein (donor) to another fluorophore attached to a binding partner (acceptor). The rate of energy transfer is dependent on the distance between the two dipoles of the donor and acceptor probes and their orientations in space. FRET is useful in mapping direct associations between proteins, and in favorable cases measuring changes in molecular proximity between the probes. This criterion will only be satisfied if the two dipoles of the donor and acceptor probes are within around 2–8 nm of each other (Foerster distance) in the protein complex and have favorable orientations. The efficiency of FRET is usually determined by measuring the quantum yield or the fluorescence lifetime of the donor probe in the absence and presence of the acceptor probe, respectively. Most high-throughput instruments record a relative value of FRET by comparing the steady-state intensity of the donor emission at a single wavelength [25]. Other measurement modes include: first, measuring changes in the intensity of the sensitized emission of the acceptor probe, which is less prone to background signal contributions; second, measuring the increase in the fluorescence polarization (FP) value of the donor, which is a consequence of the shortened lifetime of the donor probe as a result of FRET; third, measuring the decrease in the fluorescence lifetime of the donor probe as a result of FRET — this approach is usually employed in lanthanide-ion-based measurements of FRET [22]; fourth, measuring the increase in the donor emission after photobleaching the acceptor probe [26].

Because certain regulatory protein complexes form at defined locations in the cell during signaling events, the physical distributions of these events in cells can therefore be mapped based on the lifetime images of the donor probe using FLIM. As the protein complexes are formed, the quantum yield and the lifetime of the donor should decrease due to the FRET event that occurs when the acceptor in the protein complex is within the Foerster distance to the donor [8,20••,21]. Steady-state measurements of the sensitized emission resulting from FRET are easier to perform compared with the FLIM measurement of the donor lifetimes. However, it is important to note that the presence of a substantial overlap in the absorption and emission properties of the donor and acceptor probes, for example as seen in the commonly used CFP–YFP FRET pair, makes it difficult to discriminate between the emission of the acceptor probe that results from direct excitation versus sensitized emission. A very effective approach to correct this and other potential complications in CFP–YFP-based FRET imaging has recently been presented by Yue's group [20••,21].

Fluorescence polarization and fluorescence anisotropy

The hydrodynamic volume of a probe attached to a specific protein will usually increase in the larger protein complex. The hydrodynamic properties of labeled proteins can be determined by measuring the FP or FA of the emission using steady-state or time-resolved fluorescence techniques [4••,12,13,15•,27]. Measurements of FA are widely used to study interactions between proteins and drugs within high-throughput platforms [13,27]. Recent studies also show that microscope-based imaging of FA can be used to monitor protein interactions within living cells [12,28••,29,30].

Homo-FRET

Interestingly, non-radiative transfer can also occur between the dipoles of two identical probes providing they meet the same criteria outlined for hetero-FRET [8]. This occurs for probes that exhibit an overlap of their emission and absorption spectra. Homo-FRET can therefore be used to measure protein interactions with a single fluorescent probe and is especially useful in quantifying the formation of homodimers and higher oligomers of the same protein [4••,12,29,30]. However, homo-FRET does not lead to a change in the lifetime of the donor probe because the excitation energy is reversibly transferred between the same class of probe (Figure 1c, ii). In homo-FRET, the photophysical properties of the emission from a directly excited donor probe (for example, GFP), are mainly the same as those arising from the GFP-acceptor probe (Figure 1c, ii, iii) [15•,28••,29]. However, because the orientations of the dipole moments for the two interacting probes on two interacting proteins may differ, the FP value will be a function of the number of homo-FRET transfer events (Figure 1c, i). Coppey-Moisan's group recently reported a powerful, time-resolved, FA microscope technique to measure the spatial regulation of the thymidine kinase homodimeric complex using a thymidine kinase–GFP expressed within living cells [28••]. Although the homo-FRET effect is rarely used in HTS, we expect it to become more popular as it only requires a single fluorescent probe, which should prove useful in developing approaches for fluorescence-based multiplexed analysis of protein interactions.

Fluorescence correlation spectroscopy

FCS is a powerful technique for monitoring protein interactions based on the analysis of intensity fluctuation of one or a few labeled protein conjugates at nanomolar concentration in a femtoliter volume (10^{-15} l). These fluctuations depend on several factors, including the number of fluorescing species in the excitation volume and the diffusion constant of the conjugate. FCS can therefore be used to quantify rapid and reversible interactions between protein conjugates including GFP fusion

proteins without perturbing the sample or cell. While a review of the FCS literature is beyond the scope of this article, we note that changes in the fluorescence lifetime, FRET, anisotropy, spectral energy and quantum yield of fluorescent conjugates during complex formation have been exploited in FCS measurements to quantify protein interactions [6,7,31]. Finally, the introduction of microfabricated surfaces capable of limiting the illumination volume of a sample to a zeptoliter (10^{-21} l) [32] now permits FCS measurements using fluorescent conjugates at a concentration on the order of 1 μM, which corresponds to the dissociation constant found for many functional protein interactions.

Conclusion

Recent advances in proteomics and fluorescence-based detection techniques made it possible for researchers to use protein chips to identify binding partners for a specific protein within an entire proteome. In living cells, however, the activity of a given protein is often regulated through interactions within a larger complex that is transiently formed at discrete sites. Fluorescence imaging technologies outlined herein are capable of resolving these interactions. In the future, these techniques will be adapted for multiplexed analysis of protein interactions using multiple labeled proteins and images of the lifetime, polarization and energy of fluorescence from these probes. Spatio-temporal maps of specific protein interactions within a cell can be generated through these global analyses, which will help us understand the molecular regulation of important cell processes including motility and cytokinesis.

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